FEBS 29135 FEBS Letters 579 (2005) 349–356

A Gly/Ala switch contributes to high affinity binding of benzoxazinone-based non-peptide oxytocin receptor antagonists

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Received 28 June 2004; revised 24 August 2004; accepted 5 October 2004

Available online 10 December 2004

Edited by Irmgard Sinning

Abstract Non-peptide antagonists of the oxytocin receptor (OTR) have been developed to prevent pre-term labour. The benzoxazinone-based antagonists L-371,257 and L-372,662 display pronounced species-dependent pharmacology with respect to selectivity for the OTR over the V_{1a} vasopressin receptor. Examination of receptor sequences from different species identified Ala^{318} in helix 7 of the human OTR as a candidate discriminator required for high affinity binding. The mutant receptor [A318G]OTR was engineered and characterised using ligands representing many different chemical classes. Of all the ligands investigated, only the benzoxazinone-based antagonists had decreased affinity for [A318G]OTR. Molecular modelling revealed that Ala^{318} provides a direct hydrophobic contact with a methoxy group of L-371,257 and L-372,662.

Keywords: Oxytocin receptor; GPCR; Ligand binding; Non-peptide antagonist

1. Introduction

The neurohypophysial nonapeptide hormone oxytocin (OT) increases the frequency and intensity of uterine contraction at parturition [1,2]. This potent uterotonic action of OT has resulted in the peptide being used clinically to induce and to augment labour [3]. Although there is an increase in the concentration of OT in maternal plasma as pregnancy progresses, it is not consistently elevated before the onset of labour [4]. Consequently, it appears that it is not changes in hormone concentration that are critical to parturition but an increase in uterine sensitivity to OT. This increase in responsiveness is achieved through a specifically timed up-regulation of oxytocin receptors (OTRs) [5] with OTR mRNA increasing 200-fold in the myometrium at term [6]. As uterine sensitivity to OT is dictated by the abundance of OTRs available at any time, peptide [7], and more recently non-peptide [8,9], OTR antagonists have been developed to block OTRs, to induce uterine quiescence and thereby prevent the serious problem of pre-term labour [10].

The OTR has been cloned from a range of species, including human [6,11,12], it is a Family A (rhodopsin-like) G-protein-coupled receptor (GPCR) and is a member of the neurohypophysial peptide hormone receptor sub-family of GPCRs. In addition to the OTR, this GPCR sub-family includes the vasopressin receptor subtypes V_{1a} (V_{1a}R), V_{1b} (a.k.a. V_3), V_2, and the receptors for vasotocin, isotocin and mesotocin. These receptors exhibit related pharmacology [13] and possess certain sequence motifs which are characteristic of the neurohypophysial peptide hormone family of GPCRs [14]. To date, only one OTR subtype has been cloned, implying that the wide range of physiological effects of OT are mediated by a single receptor [2,6]. Significant 'cross-activation' can occur however, as OT and AVP are agonists, albeit weaker, at their counterpart receptors [13,15].

It has been shown that the OT binding platform involves extracellular domains of the OTR in addition to the transmembrane helical bundle [15–17] which is consistent with the latest OT:OTR complex molecular models [18]. Defining non-peptide antagonist recognition and mode of action at the hOTR would aid future rational drug design. However, in contrast to OT, binding contacts between non-peptide antagonists and the hOTR were unknown prior to this study.

In this study, we use species differences in the OTR:V_{1a}R selectivity of benzoxazinone-based non-peptide OTR antagonists and site-directed mutagenesis, to identify a locus in transmembrane helix 7 (TM7) of the OTR which provides a specific binding epitope required for high affinity interaction. Furthermore, subsequent molecular modelling provided the molecular mechanism underlying our observations.

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Abbreviations: GPCR, G-protein-coupled receptor; h, human; r, rat; bRho, bovine rhodopsin; OT, oxytocin; OTR, oxytocin receptor; L-366,948, {[cyclo(L-prolyl-D-2-naphthylalanyl-L-isoleucyl-D-pipecolyl-L-pipecolyl-D-histidyl)]}; L-368,899, 1-((7,7-dimethyl-2(S)-(2(S)-amino-4-(methylsulfonyl)butyramido)bicyclo[2.2.1]-heptan-1(S)-yl)-methyl)sulfonyl-4-(2-methylphenyl)piperazine; L-371,257, 1-{1-[4-[(N-acetyl-4-piperidinyl)oxy]-2-methoxybenzoyl]piperidin-4-yl}-4H-3,1-benzoxazin-2(1*H*)-one; L-372,662, 1-(1-{4-[1-(2-methyl-1-oxidopyridin-3-ylmethyl)piperidin-4-yloxyl]-2-methoxybenzoyl}piperidin-4-yl)-1,4-dihydrobenz[*d*][1,3]oxazin-2-one; AVP, [arginine^8]vasopressin; V_{1a}R, V_{1a} vasopressin receptor; InsP, inositol phosphate; $InsP_3$, inositol trisphosphate; OTA, d$(CH_2)_5$Tyr(Me)^{2}Thr^{4}Orn^{8}Tyr$(NH_2)^9$ vasotocin; TM, transmembrane helix

0014-5793/$22.00
doi:10.1016/j.febslet.2004.10.108

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), inositol-free DMEM, FBS and penicillin/streptomycin were from Gibco (Paisley, UK); cell culture plastic-ware and restriction enzyme *Esp*3I were obtained from MBI fermentas (Sunderland, UK). [Tyr^2-2,6-^{3}H]OT ([^{3}H]OT), specific activity 44.5 Ci/mmol and 2-*myo*-[^{3}H]inositol, specific activity 22.3 Ci/mmol were from PerkinElmer (Beaconsfield, UK). The peptide antagonist d[$(CH_2)_5$Tyr(Me)^{2}Thr^{4}Orn^{8}Tyr$(NH_2)^9$]VT (OTA) and OT were obtained from Bachem (UK) Ltd (St. Helens, UK). All other reagents were of analytical grade. The synthetic cyclohexapeptide antagonist L-366,948 [7] and the non-peptide OTR antagonists L-368,899 [19], L-371,257 [20] and L-372,662 [21] used in this study were synthesised at the Merck Research Laboratories (West Point, PA).

2.2. Construction of a mutant oxytocin receptor

Mutation of Ala^{318} in the hOTR was achieved using a PCR approach. The mutagenic sense oligonucleotide incorporated a single base change GCC → G**G**C (bold) for the required Ala^{318} → Gly^{318} substitution (underlined) and was 5′-C-GTC-ATG-CTC-CTG-GGC-AGC-CTG-AAT-TCC-TGC-TGC-AAC-C-3′. The PCR cycling conditions were: denaturing, 94 °C (1 min); annealing, 60 °C (2 min); extension 72 °C (1 min) for 30 cycles followed by extension at 72 °C (7 min). The A318G mutation was subcloned into the mammalian expression vector pcDNA3.1(-) containing the hOTR using unique *Bam*HI and *Esp*3I restriction sites. Mutation was confirmed by automated fluorescent sequencing (Alta Bioscience, University of Birmingham, UK).

2.3. Cell culture and transfection

HEK 293T cells were routinely cultured in DMEM supplemented with 10% (v/v) FBS, penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cells were maintained in a humidified 5% (v/v) CO_2 incubator at 37 °C. HEK 293T cells were seeded at a density of approximately 5×10^5 cells/100 mm dish, and transfected after 48 h using the calcium phosphate precipitation method. Briefly, a DNA-calcium phosphate co-precipitate, containing 10 μg plasmid cDNA for each dish, was prepared 30 min before use. After incubation for 16 h, the media was replaced with growth media and cells incubated for 48 h before harvesting.

2.4. Radioligand binding assays

Preparation of HEK 293T cell membranes for subsequent radioligand-receptor binding assays was as described previously [22]. The protein concentration of membranes was determined using the Pierce BCA protein assay kit (Pierce Chemical Co.) with BSA as standard. Binding assays were essentially as described previously [23,24] and contained membranes (200–420 μg), [^{3}H]OT (0.8–2.2 nM) as tracer and competing ligand at the concentrations indicated, in a final volume of 0.5 ml. Non-specific binding was defined by unlabeled OT (10 μM). After incubation for 90 min at 30 °C to establish equilibrium, bound and free ligand were separated by sedimentation at $12\,000 \times g$ for 10 min. Membrane pellets were washed and solubilised using Soluene-350 (PerkinElmer) prior to liquid scintillation counting using Hi-Safe3 (PerkinElmer) as cocktail. Radioligand binding results are expressed as a percentage of the control (i.e., specific binding in the absence of the competing ligand). IC_{50} values were determined by non-linear regression after the fitting of Langmuir binding isotherms to experimentally determined data using the Fig. P. Program (Biosoft, Milltown, NJ, USA). Dissociation constants (K_d) were calculated from IC_{50} values as described [25], using the experimentally determined value for the affinity of [^{3}H]OT for each construct. Differences between the affinity of ligands for wild-type and [A318G]OTR were evaluated for statistical significance by Student's *t* test.

2.5. Measurement of OT-induced accumulation of inositol phosphates

HEK 293T cells were seeded at a density of 2.5×10^5 cell/well in poly-*D*-lysine-pre-treated twelve-well plates. OT-induced accumulation of inositol phosphates was assayed in transfected cells as described previously [26,27]. Essentially, 24 h post-transfection, medium was replaced with inositol-free, DMEM containing 1% (v/v) FBS and 2.0 μCi/ml 2-*myo*-[^{3}H]inositol. After 24 h at 37 °C, cells were washed twice with PBS and incubated with medium containing 10 mM LiCl for 30 min, followed by the addition of OT at the concentration indicated for a further 30 min. Incubations were terminated by adding 0.5 ml of 5% (v/v) of perchloric acid containing 1 mM EDTA, and 1 mg/ml phytic acid hydrosylate. Cells were pelleted at $12\,000 \times g$ for 5 min, supernatants neutralised on ice with 1.2 M KOH, 10 mM EDTA, 50 mM HEPES, and samples loaded onto 0.8 ml Bio-Rad AG1-X8 columns (formate form). After elution of inositol (10 ml water) and glycerophosphoinositol (10 ml, 25 mM NH_4COOH containing 0.1 M HCOOH), a mixed inositol phosphate fraction containing inositol mono-, bis-, tris-phosphates was eluted (10 ml, 850 mM NH_4COOH containing 0.1 M HCOOH). Radioactivity was determined using Ultima-Flo AF scintillant (PerkinElmer). EC_{50} values were determined by non-linear regression after fitting of logistic sigmoidal curves to the experimental data.

2.6. Molecular modelling of the hOTR

An hOTR homology model was generated from the ground-state crystal structure of bovine rhodopsin (bRho) determined at 2.8 Å resolution [28] (PDB accession numbers 1F88, 1HZX). An alignment between bRho and the hOTR was made with Fasta followed by hand adjustment. Overall sequence similarity between the hOTR and rhodopsin is 48% when identical residues and conserved substitutions are included. The model was generated using the homology modelling module in InsightII from MSI. Energy minimisation of the receptor model was carried out to relieve steric repulsion of the side-chains, while Cα of the transmembrane helices were constrained so that the backbone of the TM regions remained unchanged. The loops were further refined with high temperature molecular dynamic simulations. A database of 100 different conformations of each of the non-peptide antagonists L-371,257, L-372,662 and L-368,899 was prepared which incorporated geometric constraints for the position of atoms within the molecule, such as bond length and bond angle, together with allowed torsion angles. The conformations generated were then energy minimised. The ligand binding site of the OTR was defined as the region within 10 Å of the position rhodopsin-bound retinal would occupy when projected onto the OTR structure, a position supported by empirical site-directed mutagenesis data [29]. A grid map was generated which described the energetics of the binding site. Each of the different conformations was docked into the binding site using the docking program FLOG (flexible ligand on a grid) with reference to the grid map. Approximately thirty high-ranking binding conformations of each compound were visually inspected. Poses exhibiting steric clashes with the receptor were discounted, whereas those possessing reasonable van der Waals contacts and good polar interactions were considered candidates. When each of the candidate poses of the docked ligands was compared for all of the compounds investigated, a consensus conformation emerged for L-371,257 and L-372,662. In contrast, L-368,899 which possesses a bulky group and a sulfate in the centre of the molecule, did not adopt a conformation similar to this consensus conformation.

3. Results

3.1. Pharmacological characterisation of hOTR constructs

The structurally-related non-peptide oxytocin antagonists L-371,257 and L-372,662 (Fig. 1) are capable of binding to both OTRs and V_{1a}Rs [20,21]. However, the interaction of L-371,257 and L-372,662 with these two receptors differs in one important aspect. Although these ligands bind with high affinity to the OTR from both human and rat, they exhibit species-specific pharmacology with respect to the V_{1a}R. For example, L-371,257 displays high affinity for the rat V_{1a}R (K_d = 3.7 nM) but low affinity for the human V_{1a}R (K_d = 3200 nM [20]). This implies that residues that are conserved in the hOTR, rOTR and rV_{1a}R but which are substituted in the hV_{1a}R are candidates for providing a high affinity binding epitope for L-371,257 and L-372,662. Comparison of the sequence of TM7 of the hOTR, rOTR, hV_{1a}R and rV_{1a}R revealed a high degree

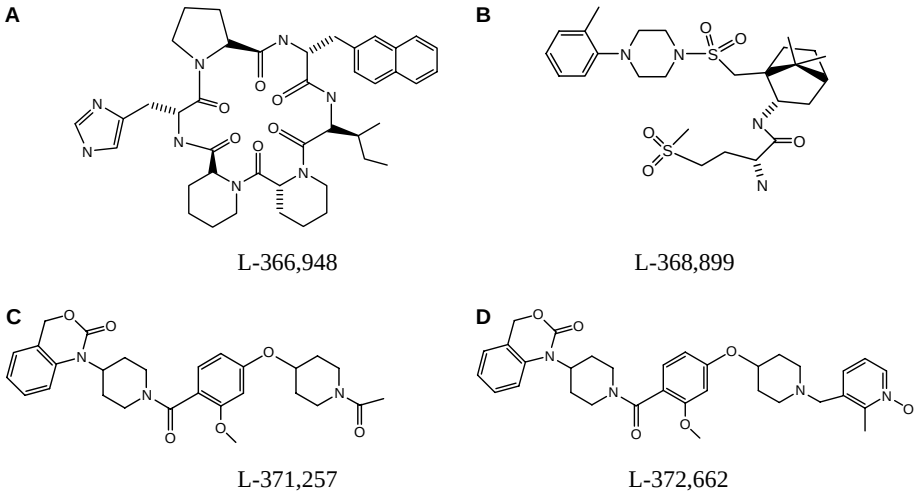

Fig. 1. Structure of OTR antagonists. The structures of antagonists used in this study are shown. (A) L-366,948; (B) L-368,899; (C) L-371,257 and (D) L-372,662.

of sequence homology between these three receptors with 70% absolute identity within this helix. It was noted (Fig. 2), that residue 7.42 (using the notation proposed for GPCRs by Ballesteros and Weinstein [30]) is an alanyl in the hOTR, rOTR and the $rV_{1a}R$ (Ala^{318}, Ala^{317}, Ala^{342}, respectively) but is replaced by a glycyl (Gly^{337}) in the $hV_{1a}R$, and is therefore a candidate for providing a binding contact required for high affinity binding of the non-peptide antagonists L-371,257 and L-372,662.

To investigate the role of residue 7.42 in non-peptide ligand recognition, a mutant hOTR was engineered in which Ala^{318} in the hOTR was substituted by Gly^{318} ([A318G]OTR). The construct [A318G]OTR was expressed in HEK 293T cells, pharmacologically characterised and compared to wild-type hOTR. Both receptors were expressed at the same level of 0.2–0.5 pmol/mg protein. A range of ligands representing very diverse structural classes was employed for this pharmacological characterisation (Fig. 1). These were, (i) the natural peptide agonist OT; (ii) the peptide antagonist $(CH_2)_5Tyr(Me)^2Thr^4Orn^8Tyr^9NH_2$ (OTA); (iii) the synthetic cyclohexapeptide antagonist L-366,948 (Fig. 1A) [7]; (iv) the camphor-based non-peptide antagonist L-368,899 (Fig. 1B) [19,31] and (v) the benzoxazinone-based non-peptide antagonists L-371,257 (Fig. 1C) [20] and L-372,662 (Fig. 1D) [21]. Competition radioligand binding curves were determined for each ligand binding to [A318G]OTR and to the wild-type hOTR (Fig. 3). The K_d values are presented in Table 1, corrected for radioligand occupancy. The affinity of OT and the three classes of antagonist represented by OTA, L-366,948 and L-368,899 were unchanged by the Ala^{318} to Gly^{318} substitution. In contrast, both of the benzoxazinone-based non-peptide antagonists (L-371,257 and L-372,662) were affected (Fig. 3E and F) and exhibited a 6–12-fold lower affinity for [A318G]OTR compared to wild-type hOTR (Table 1). The wild-type binding characteristics of [A318G]OTR with respect to the natural agonist and three different chemical classes of antagonist (both peptide and non-peptide) was strong evidence that the overall folding of the receptor had not been perturbed by the $Ala^{318} \rightarrow Gly^{318}$ (7.42) mutation in TM7. Further evidence that wild-type tertiary structure was preserved in [A318G]OTR was provided by the observation that the dose–response relationship for OT-induced inositol phosphates accumulation by [A318G]OTR was wild-type (Fig. 4).

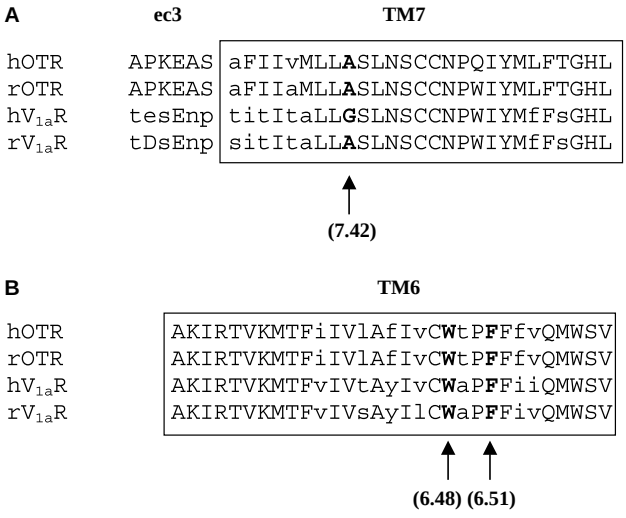

Fig. 2. Alignment of the TM7 helix of the OTR and $V_{1a}R$ from human (h) and rat (r). Residues absolutely conserved in all four receptors are shown in capital letters. Sequences cited were obtained from Swissprot PDB and GenEMBL. Panel A: the distal segment of the third extracellular loop (e3) is shown with the TM7 helix (boxed) in each case. The position of residue (7.42) is indicated by an arrow and bold typeface. Panel B: the TM6 helix (boxed) is shown in each case. The position of residues Phe (6.51) and Trp (6.48) is indicated by an arrow and bold typeface.

3.2. Molecular modelling of non-peptide antagonists docked to hOTR

An homology model of the hOTR was constructed utilising the recently solved crystal structure of bRho [28]. Both of these receptors belong to the Family A class of GPCRs and exhibit a series of highly conserved 'signature' residues in the transmembrane regions (shown in bold in Fig. 5) which are characteristic of this family of receptors. The alignment preserves the relative

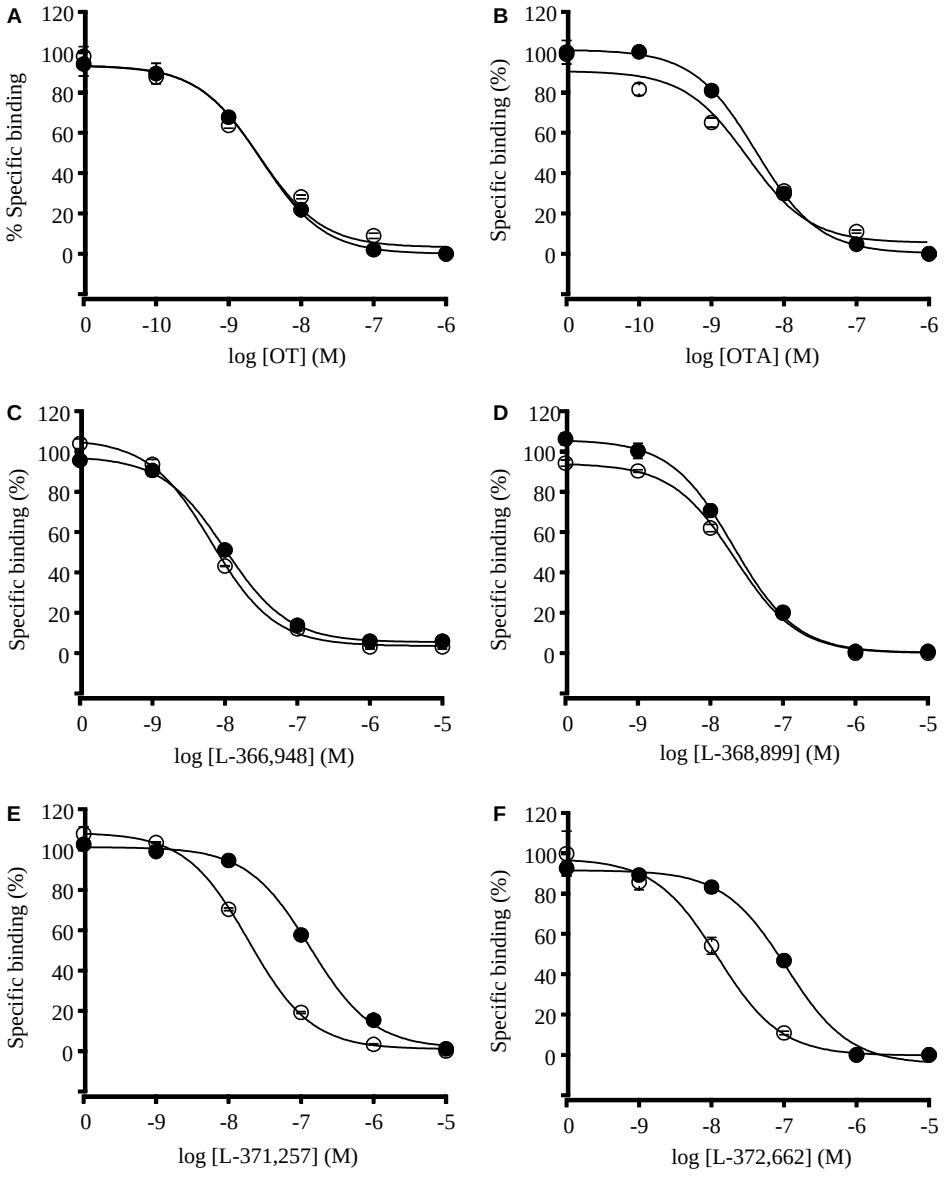

Fig. 3. Pharmacological characterisation of wild-type hOTR and [A318G]OTR. Competition radioligand binding studies using HEK 293T cells expressing either the wild-type OTR (○) or the [A318G]OTR mutant (●) were performed using (A) OT; (B) peptide antagonist, OTA; (C) cyclopeptide antagonist, L-366,948; (D) the camphor-based non-peptide antagonists L-368,899 and the benzoxazinone-based non-peptide antagonists L-371,257 and L-372,662, (E) and (F), respectively. Data shown are the means ± S.E.M. of at least three separate experiments each performed in triplicate. Values are expressed as % specific binding where non-specific binding was defined by OT (10 μM). A theoretical Langmuir isotherm competition curve was fitted to the experimental data as described in Section 2.

positions of these 'signature' residues in bRho and OTR. Non-peptide ligands were then docked, as described in Section 2. From the binding positions of L-371,257, L-372,662 and L-368,899 presented in Fig. 6A, B and C, respectively, it is apparent that all of these compounds occupy a similar region within the transmembrane helical bundle of the hOTR. In the wild-type hOTR, Ala^{318} (7.42) is located in direct hydrophobic contact with the methoxy group protruding from the benzene moiety which is central to the structure of L-371,257 (Fig. 6A). It can be seen that Ala^{318} (7.42) straddles two aromatic rings, with Phe^{291} (6.51) and Trp^{288} (6.48) positioned above and below, respectively. This orientation enables these two residues in TM6 to make π-stacking interactions with the same central benzene ring of L-371,257 contacted by Ala^{318}. From this molecular arrangement it is apparent that mutation of the Ala^{318} in the construct [A318G]OTR would result in the loss of the hydrophobic contact between the methoxy of L-371,257 and residue 7.42 of the receptor (Fig. 6A). Furthermore, in the absence of the Ala^{318} to influence local side-chain orientation, there would be free rotation of the side-chains of Phe^{291} and Trp^{288}, resulting in a corresponding decrease in π-stacking interactions with the benzene group of L-371,257. Modification of the acetylpiperidine terminus of L-371,257 by pyridine *N*-oxide resulted in the compound L-372,662 (Fig. 1D). From the overlay presented in Fig. 6B it is apparent that both L-371,257 and L-372,662 establish very similar interactions when bound to the hOTR. Consequently, the role of Ala^{318} and the molecular ramifications of the A318G mutation

Table 1
Pharmacological profile of wild-type hOTR and [A318G]OTR

Ligand	Binding affinities K_d (nM)	
	OTR	A318G
OT	1.4 ± 0.5	1.4 ± 0.3
OTA	1.7 ± 0.5	2.4 ± 0.4
L-366,948	4.0 ± 0.7	5.4 ± 0.8
L-368,899	14 ± 1.9	13 ± 2.2
L-371,257	12 ± 0.8	72 ± 13 (4)***
L-372,662	5.8 ± 0.4	73 ± 23 (4)***
Expression B_{max} (pmoles/mg protein)	0.5 ± 0.2	0.2 ± 0.1

***Significantly different from wild-type OTR, $P < 0.001$.
[^{3}H]OT was used as tracer in competition radioligand binding assays. Dissociation constants (K_d) were calculated from IC_{50} values, corrected for radioligand occupancy. All values shown are the means ± S.E.M. of three separate experiments, except where $n = 4$ is indicated, each performed in triplicate.

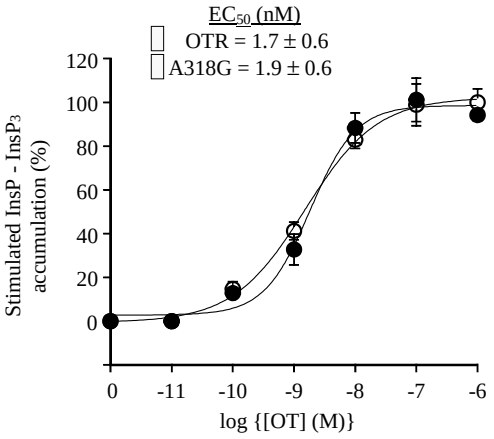

Fig. 4. Intracellular signalling by wild-type hOTR and [A318G]OTR. OT-induced accumulation of InsP–InsP$_3$ in HEK 293T cells transfected with either wild-type OTR (○) or A318G mutant (●). Data shown are the means ± S.E.M. of three separate experiments performed in triplicate. Values are expressed as % maximum stimulation induced by OT at the stated concentrations.

are the same for both L-371,257 and L-372,662. The pyridine moiety of L-372,662 is accommodated within an hydrophobic pocket formed by the residues Val^{88} and Val^{93} of TM2 in conjunction with Ile^{49} and Ala^{53} of TM1 while the *N*-oxide is in close contact with Ser^{322} of TM7 and forms a hydrogen bond (Fig. 6B). This additional interaction may explain why the affinity of L-372,662 for the hOTR is slightly higher than that of L-371,257 (Table 1). The camphor-based non-peptide antagonist L-368,899 also binds in the vicinity of Ala^{318}, Phe^{291} and Trp^{288}. However, in this instance there is no direct hydrophobic contact between the ligand and Ala^{318} and the interaction of Phe^{291} and Trp^{288} with L-368,899 is less optimal than with L-371,257 due to the lack of aromaticity and the overall difference in conformation (Fig. 6C). The absence of a binding contact between L-368,899 and Ala^{318} explains why, in marked contrast to the benzoxazinone-based antagonists L-371,257 and L-372,662, this camphor-based antagonist binds with high affinity to both wild-type hOTR and the mutant construct [A318G]OTR.

4. Discussion

The non-peptide ligand L-371,257 was developed as an OTR antagonist from the structure of the $V_{1a}R$ non-peptide antagonist OPC-21268 [32] as the lead compound [9,20]. Subsequent characterisation revealed that L-371,257 exhibits species-specific pharmacology, which has profound ramifications with

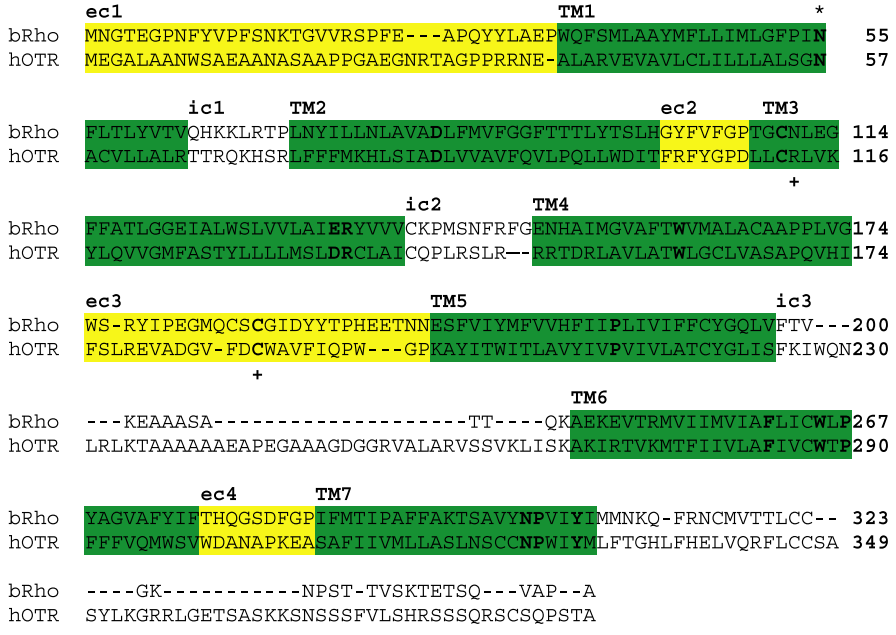

Fig. 5. Alignment of the sequence of bRho and the hOTR. Transmembrane (TM) regions are shown in green, extracellular (ec) and intracellular (ic) loops are shown in yellow and white, respectively. Residue number is indicated on the right-hand side of the figure. Residues conserved throughout Family A GPCRs are indicated in bold and cysteines forming a disulphide bond in Family A GPCRs are indicated by '+'.

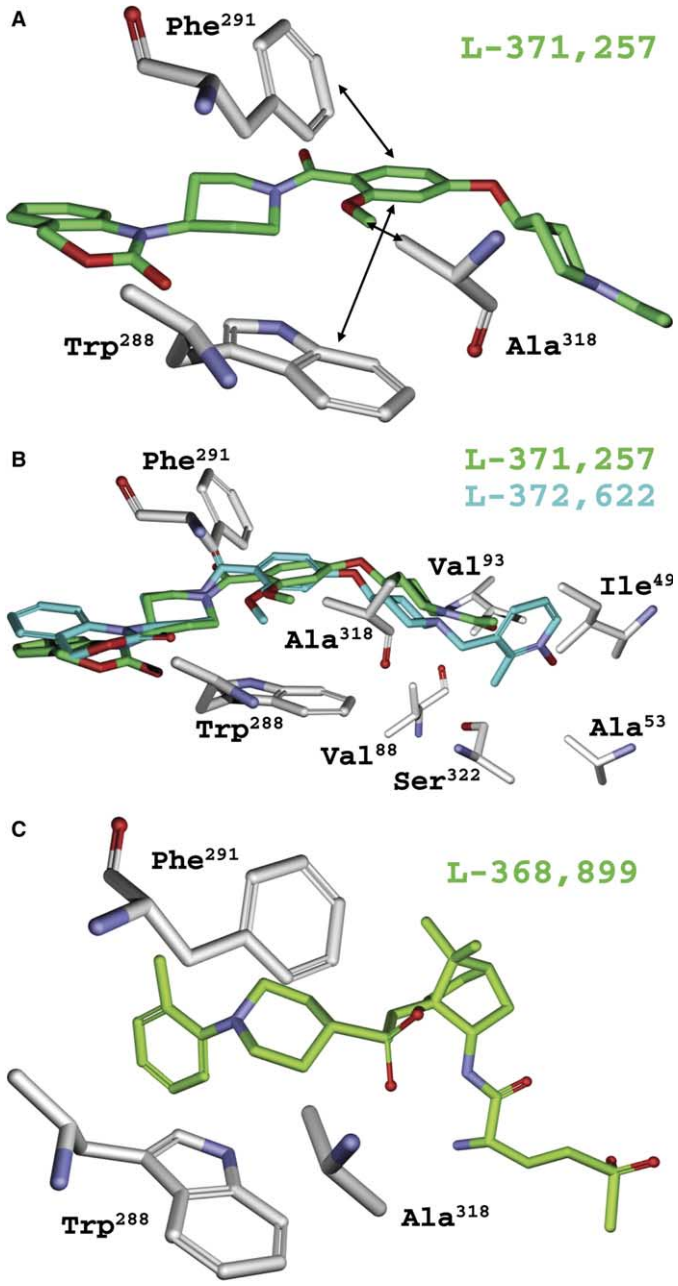

Fig. 6. Molecular model of the hOTR docked with non-peptide antagonists. Amino acid side-chains are shown in white and are labelled in yellow together with the residue number. In each case, only key residues cited in the text are illustrated for clarity. (A) Interaction of L-371,257 with Ala^{318} (7.42), Phe^{291} (6.51) and Trp^{288} (6.48). The contacts formed when the benzoxazinone-based non-peptide antagonist L-371,257 is bound to the hOTR are indicated by double-headed arrows. (B) Overlay of L-371,257 (green) and L-372,662 (cyan) docked to the hOTR revealing the high degree of overlap between the binding position of these structurally-related ligands and the common role of Ala^{318}. (C) Although the camphor-based non-peptide antagonist L-368,899 binds in a similar position in the hOTR as the benzoxazinone-based non-peptide antagonists L-371,257 and L-372,662 it does not make the same binding contacts and does not involve Ala^{318}. For further details refer to the text.

respect to its potential therapeutic applications. Consequently, L-371,257 is slightly V_{1a}R-selective in the rat (5-fold) but in marked contrast, it is profoundly OTR-selective in the human (865-fold) [8,20]. Given the therapeutic potential of L-371,257, the compound has been subjected to extensive and systematic chemical modification aimed at improving its pharmacological characteristics as a putative drug for the treatment of pre-term labour. One such series, incorporating various pyridine *N*-oxide groups at the acetylpiperidine terminus of L-371,257, generated L-372,662 which exhibited increased potency, improved pharmacokinetics and excellent bioavailability [21]. The pronounced species-dependent pharmacology with respect to OTR:V_{1a}R selectivity was exhibited by both of the benzoxazinone-based compounds L-371,257 and L-372,662. Such species-selectivity is not restricted to non-peptide ligands however, as the phenomenon has also been reported for a range of neurohypophysial hormone receptor peptide ligands [33–35]. Correlating the pharmacological characteristics of L-371,257 and

L-372,662 with sequence homology between OTR and $V_{1a}R$ from human and rat, identified Ala^{318} (7.42) in the hOTR as a candidate for providing a species-specific binding epitope for these non-peptide antagonists.

The binding affinity of L-372,662 was decreased by the $Ala^{318} \rightarrow Gly^{318}$ mutation in a very similar manner to the lead compound L-371,257 (Fig. 3E and F), despite the structural differences (Fig. 1). This is entirely consistent with our molecular model, as the features of L-371,257 which are shown in Fig. 6A to be important for interacting with the receptor in the L-371,257:hOTR complex are retained in L-372,662 (Fig. 1). Consequently, the model predicted that Ala^{318} (7.42) contributed to high affinity binding of both L-371,257 and L-372,662 to the hOTR (Fig. 6B). Although Ala^{318} was required for high affinity binding of benzoxazinone-based non-peptide antagonists, it did not provide binding epitopes for non-peptide antagonists in general (Fig. 3 and Table 1). Modelling of L-371,257 and L-372,662 plus another chemically-unrelated non-peptide antagonist L-368,899, docked to the hOTR provided the molecular explanation for these observations (Fig. 6). Our data indicate an interaction between the benzoyl methoxy group of the ligand L-371,257 and Ala^{318} (7.42) of the hOTR. This proposed interaction of Ala^{318} with a methoxy group in the ligand is supported experimentally. Removal of the methoxy group from the ligand, to generate the des-methoxy analogue of L-371,257, resulted in a 4-fold reduction in affinity for the hOTR [20]. This is almost identical to the 6-fold decrease in affinity of L-371,257 for the hOTR resulting from the A318G mutation (Table 1). Taken together these two observations are supportive of a role for Ala^{318} (7.42) in binding L-371,257. Although Ala^{318} (7.42) provides a molecular discriminator underlying species-selective binding of benzoxazinone-based antagonists with OTRs, this would obviously be only one of many contacts between the docked ligand and the receptor, the majority of which do not contribute to species-specific pharmacology.

Ala^{318} did not have a role in binding peptides (agonist or antagonist), nor in the OT-induced transition from the ground state of the receptor (R) to the active conformation (R*) as intracellular signalling of [A318G]OTR was wild-type (Fig. 4). It has recently been reported by two groups independently, that high affinity binding of the non-peptide antagonist OPC-21268 to the $V_{1a}R$ also required an alanyl in TM7 at residue 7.42 [36,37], whereas the binding of the non-peptide antagonist SR 49059 to the $V_{1a}R$ did not [36]. The proposed orientation of OPC-21268 binding to the $hV_{1a}R$ [38] however, is different from that of L-371,257 docked to the hOTR (Fig. 6A). With respect to the binding of the benzoxazinone-based antagonists L-371,257 and L-372,662 to the $V_{1a}R$, the Ala (7.42) is preserved in the rat receptor which exhibits high affinity, but is replaced by a Gly (7.42) in the human receptor which exhibits low binding affinity (Fig. 2A). Furthermore, the aromatic side-chains of Phe (6.51) and Trp (6.48) in TM6, which contact the central benzene ring of L-371,257 and straddle the Ala (7.42) in the hOTR, are also conserved in $V_{1a}Rs$ (Fig. 2B). Consequently, key features identified for high affinity binding of benzoxazinone-based antagonists to the hOTR are also present in the $rV_{1a}R$ which also exhibits high affinity binding of L-371,257 and L-372,662.

It is noteworthy that when docked into the hOTR binding site, L-371,257 and L-372,662 reside in a similar location in the TM helical bundle as the retinal binding site in bRho. Indeed, the Ala^{318} (7.42) which contributes to the high affinity binding of these compounds is only one residue higher in the TM7 helix than the 11-*cis*-retinal covalent attachment site in rhodopsin (Lys^{296} (7.43)).

In conclusion, this study provides a plausible molecular model for the species-dependent OTR:$V_{1a}R$ selectivity exhibited by benzoxazinone-based non-peptide antagonists.

Acknowledgement: Financial support for this work was provided by grants to M.W. from the Biotechnology and Biological Sciences Research Council. We are grateful to Dr. Tadashi Kimura (University of Osaka, Japan) for providing the hOTR clone and to Dr. S.A. White (University of Birmingham, UK) for assistance in preparing Fig. 6 for publishing. We also thank Drs. Peter Williams, Ian Bell and Roger Freidinger (Merck Research Laboratories, West Point, PA) for providing samples of the OTR antagonists L-366,948, L-368,899, L-371,257 and L-372,662 for these studies.

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